

## Enamel Matrix Derivative Stimulates Osteogenesis- and Chondrogenesis-related Transcription Factors in C3H10T1/2 Cells

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**Abstract** Our purpose was to determine how enamel matrix derivative (EMD) affects the expression of osteogenesis- and chondrogenesis-related transcription factors in undifferentiated mesenchymal cells. C3H10T1/2 cell line, a typical pluripotential mesenchymal cell line, was cultured with or without EMD for up to 7 d. Expression of mRNAs encoding osteogenesis- and chondrogenesis-related transcription factors (Runx2, Osterix, AJ18, Dlx5, Msx2, Sox5, Sox9 and Zfp60) was measured using real-time polymerase chain reaction. Runx2 and Sox9 protein expression and the presence of bone morphogenetic protein (BMP)-6-like molecules in EMD were determined by Western blotting. EMD substantially increased mRNA levels of osteogenesis- and chondrogenesis-related transcription factors. EMD also induced Runx2 and Sox9 protein expression. Western blotting analysis of EMD using anti-BMP-6 antibody revealed immunoreactive bands corresponding to about 14 kDa and 60 kDa. These results suggest that EMD stimulates osteogenesis- and chondrogenesis-related transcription factors, and these activities may be mediated, at least in part, by BMP-6 in EMD.

**Key words** enamel matrix derivative; osteogenesis; chondrogenesis

One of the most important aspects of successful periodontal treatment is the regeneration of multiple periodontal tissues accompanying the newly formed cementum and alveolar bone [1,2]. Enamel-related matrix proteins secreted by Hertwig's epithelial root sheath cells induce root formation and cementogenesis [3,4]. In 1997, enamel matrix derivative (EMD, also named as Emdogain) obtained from embryonic enamel matrix extracts became commercially available. This product has been widely used in clinical settings to promote the formation of both cementum and alveolar bone [5]. *In vitro* studies have demonstrated that EMD stimulates proliferation of human periodontal ligament-derived cells, as well as protein

synthesis and mineralization by these cells [6]. Furthermore, EMD also stimulates the differentiation of primary osteoblasts and osteoblastic cell lines [7,8].

We previously reported that EMD diverts the pluripotent mesenchymal cell line C2C12 to an osteogenic and/or chondrogenic differentiation pathway [9]. More recently, we demonstrated that EMD increases expression of runt-related transcription factor-2 (Runx2) and induces phosphorylation of mothers against decapentaplegic homolog 1 (Smad1) [10], and proposed that these effects were mediated by bone morphogenetic protein (BMP)-2/4-like or BMP-7-like molecules present in EMD. However, the mechanisms that regulate the transcription level remain poorly understood.

In the present study, we examined the effect of EMD on osteogenesis- and chondrogenesis-related gene expression and sought to identify the EMD components involved in osteogenic and/or chondrogenic lineages of

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C3H10T1/2 cells.

## Materials and Methods

### Cell culture

The mouse fibroblastic cell line C3H10T1/2 was obtained from the Riken Cell Bank (Tsukuba, Japan). The cells were maintained in a growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, Rockville, USA) containing 10% (V/V) fetal bovine serum (FBS) (HyClone Laboratories, Logan, USA) and 1% (V/V) penicillin-streptomycin solution (50 U/ml penicillin and 50 µg/ml streptomycin) (Sigma, St. Louis, USA). Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. For the treatment with EMD (Biora AB, Malmö, Sweden), the cells were seeded onto tissue culture plates at a density of 1×10<sup>4</sup> cells/cm<sup>2</sup>. After an overnight incubation, the cells were cultured for up to 7 d in DMEM containing 10% FBS and 0, 10, 50 or 100 µg/ml EMD.

### Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cultured C3H10T1/2 cells using the RNeasy mini kit (Qiagen, Valencia, USA). Aliquots of equal amounts of mRNA were subjected to real-time PCR. First-strand cDNA synthesis was carried out with the GeneAmp RNA PCR kit (PerkinElmer, Branchburg, USA). The cDNA mixtures were diluted 5-fold in sterile distilled water, and 2-µl aliquots were subjected to real-time PCR using SYBR green I dye. The reactions were performed in 25 µl of SYBR premix Ex *Taq* solution (TaKaRa Shuzo, Shiga, Japan) with specific primers as

shown in **Table 1**. PCR was performed in a thermal cycler (Smart Cycler; Cepheid, Sunnyvale, USA), and the data were analyzed using Smart Cycler software 1.2d. The PCR conditions were 3 s at 95 °C, 20 s at 68 °C, 40 cycles. The measurement was taken after each cycle. PCR product specificity was verified by melting curve analysis between 68 °C and 94 °C. All real-time PCR reactions were performed in triplicate, and the levels of mRNA expression were calculated and normalized to the mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at each time point.

### Extraction of protein from cultured cells

To obtain whole-cell extracts, C3H10T1/2 cells that had been incubated with EMD were rinsed with phosphate-buffered saline (PBS) and then exposed to a lysis buffer consisting of 50 mM Tris-HCl, 0.1% Triton X-100, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Cells in the lysis buffer were sonicated three times for 10 s each time. Aliquots that contained equal amounts of protein were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

### SDS-PAGE and Western blotting

SDS-PAGE was conducted at 150 V for 60 min in 5%–20% gradient gels using a discontinuous Tris-glycine buffer system, as described by Laemmli [11]. Immunotransfer was carried out using a semidry transfer unit with a continuous buffer system at 0.8 mA/cm<sup>2</sup> for 60–90 min. The membrane was blocked at 4 °C for 16 h, and then washed with Tris-buffered saline containing Tween 20 (TBS-T). For immunodetection, the blocked membrane was incubated at room temperature for 90 min with anti-Runx2, anti-Sox9, anti-BMP-6 and anti-β-tubulin antibodies.

**Table 1** Primers used in real-time polymerase chain reaction

Target	Forward primer (5'→3')	Reverse primer (5'→3')	GenBank accession No.
Runx2	GCGTCAACACCATCATTCTG	CAGACCAGCAGCACTCCATC	NM_004348
Osterix	GGAGGTTTCACTCCATTCCA	TAGAAGGAGCAGGGGACAGA	AF184902
Dlx5	GCGCTCAACCCATAACCAGT	ACTCGGGACTCGGTTGTAGG	AB073716
Msx2	TCACCACGTCCCAGCTTCTAG	AGCTTTTCCAGTCCGCCTCC	NM_013601
AJ18	CCCCAAGGAAGTCACCAGT	CTTTCTATGGGATCGGTCTCTT	AF321874
Sox5	ATGGTGTGGGCGAAACATGA	GGCGGGCCTGCTCCT	AB006330
Sox9	ATCTGAAGAAGGAGAGCGAG	TCAGAAGTCTCCAGAGCTTG	AF421878
Zfp60	CGTCTTACTAGAGCCGGAGAAA	ACTTACAACCAAAGCACTTCCC	U48721
Osteocalcin	GACAAGTCCCACACAGCAACT	GGACATGAAGGCTTTGTGTCAGA	L24431
Type II collagen	ATGACAATCTGGCTCCCAAGACTGC	GACCGGCCCTATGTCCACACCCGAAT	NM_001844
GAPDH	GAGTCAACGGATTTGGACGT	GACAAGCTTCCCGTTCTCAG	NG_003011

ies (Santa Cruz Biotechnology, Santa Cruz, USA), then washed in TBS-T. The membrane was then incubated at room temperature for 60 min with appropriate biotin-conjugated secondary antibodies, washed with TBS-T, and incubated for 20 min at room temperature with horseradish peroxidase (HRP)-conjugated streptavidin. Immunoreactive proteins were visualized using a commercially available chemiluminescence ECL kit (Amersham Biosciences, Buckinghamshire, UK) with exposure of the transfer membrane to X-ray film (Eastman Kodak, Rochester, USA).

### Statistical analysis

All data are shown as the mean $\pm$ SD. Statistical significance was determined using Bonferroni's modification of the Student's *t*-test. Results were considered statistically significant at  $P < 0.05$ .

## Results

### mRNA levels of osteogenesis- and chondrogenesis-related genes

The expressions for osteogenesis- and chondrogenesis-

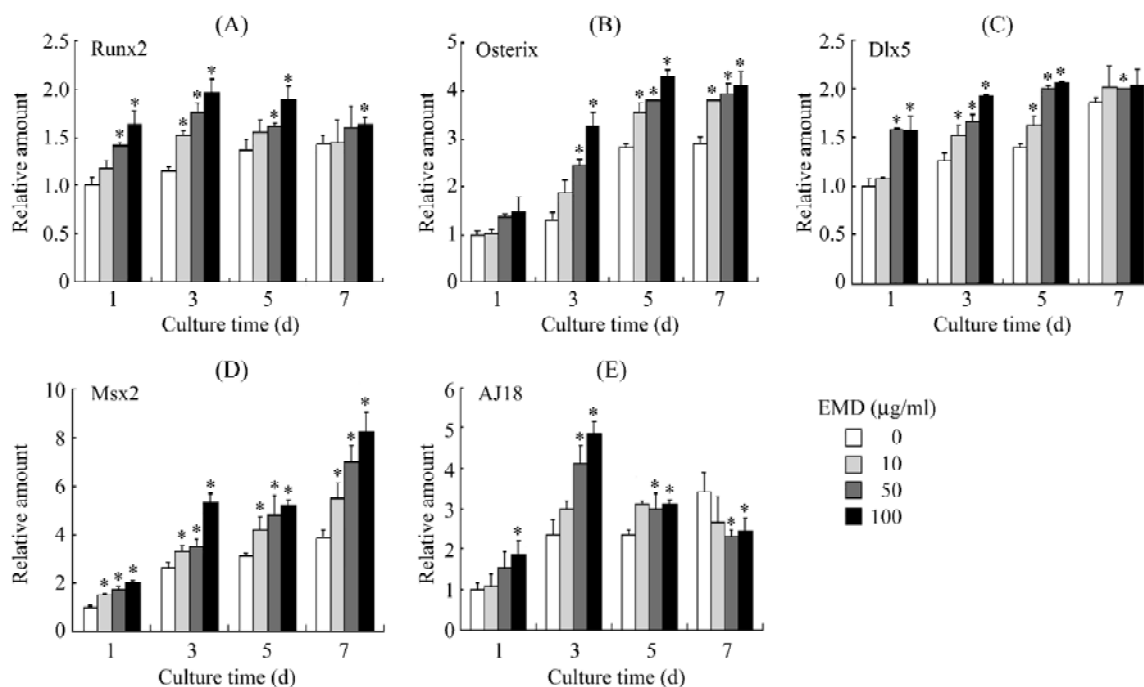
related genes were determined by using real-time PCR analysis on day 1, 3, 5 and 7, respectively, in cells cultured in medium containing 0, 10, 50 or 100  $\mu$ g/ml EMD. Five days of EMD treatment significantly increased the level of Runx2 mRNA compared with the control [Fig. 1(A)]; EMD also significantly increased the level of Osterix mRNA on day 3–7 [Fig. 1(B)], and the level of Dlx5 mRNA [Fig. 1(C)] and Msx2 mRNA [Fig. 1(D)] on day 1–7; and EMD transiently enhanced the level of AJ18 mRNA, reaching a maximum on day 3 then decreasing on day 7 [Fig. 1(E)].

A high concentration of EMD induced the expression of Sox5 [Fig. 2(A)], Sox9 [Fig. 2(B)] and Zfp60 [Fig. 2(C)] during the culture period.

### Effect of EMD on Runx2 and Sox9 protein expression

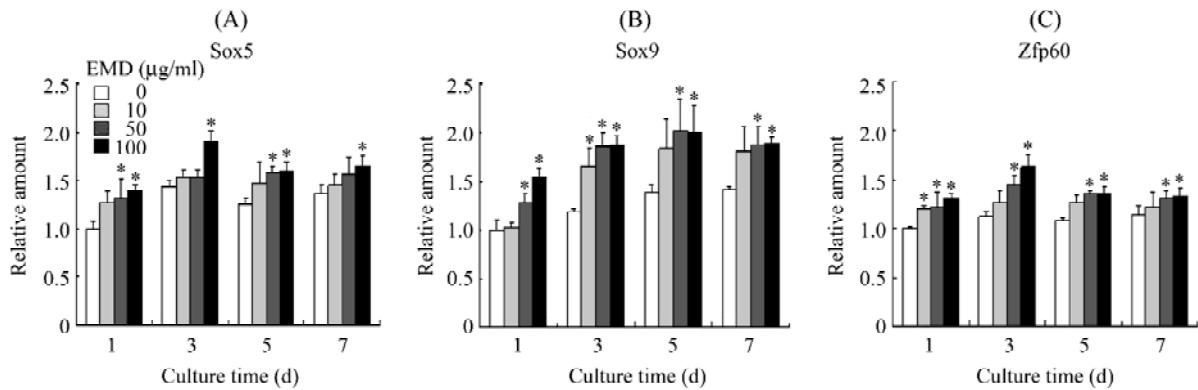
Using Western blotting analysis, immunoreactive bands running at about 55 kDa and 65 kDa were generated in response to anti-Runx2 and anti-Sox9 antibodies, respectively, as shown in Fig. 3. The amount of protein in these bands increased substantially in response to EMD in a dose-dependent manner.

### Effect of EMD on osteocalcin and type II collagen mRNA expression



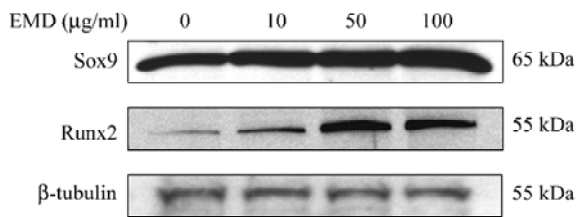
**Fig. 1** Effect of enamel matrix derivative (EMD) treatment on the expression of osteogenesis-related genes

C3H10T1/2 cells were cultured with 0, 10, 50, or 100  $\mu$ g/ml EMD for up to 7 d. On day 1, 3, 5, and 7, the expression of osteogenesis-related mRNA was determined using real-time polymerase chain reaction, as indicated. The data shown are the mean $\pm$ SD for three separate experiments. \* $P < 0.05$  EMD-treated versus corresponding control group.



**Fig. 2** Effect of enamel matrix derivative (EMD) treatment on the expression of chondrogenesis-related genes

C3H10T1/2 cells were cultured with 0, 10, 50, or 100 µg/ml EMD for up to 7 d. On day 1, 3, 5 and 7, the expression of chondrogenesis-related mRNA was determined using real-time polymerase chain reaction, as indicated. The data shown are the mean±SD for three separate experiments. \* $P$ <0.05 EMD-treated versus control.



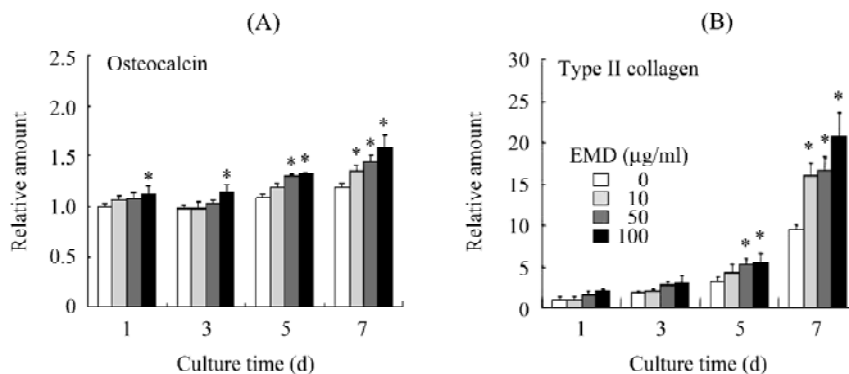
**Fig. 3** Effect of enamel matrix derivative (EMD) treatment on Runx2 and Sox9 protein expression

Nuclear protein was isolated from C3H10T1/2 cells that had been incubated with various concentrations of EMD for 5 d. Runx2 and Sox9 expression was analyzed by Western blotting.

On the basis of the real-time PCR analysis, a high concentration of EMD induced osteocalcin [Fig. 4(A)] and type II collagen mRNA [Fig. 4(B)] on day 1–3. EMD also significantly increased these genes in a similar dose-dependent manner on day 5–7.

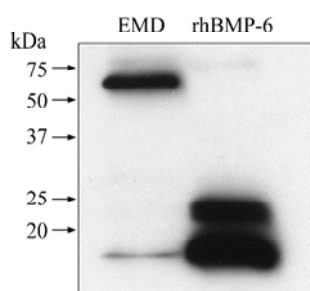
#### BMP-6-like molecules in EMD

Using Western blotting analysis, immunoreactive bands running at about 14 kDa and 60 kDa were generated in response to the anti-BMP-6 antibody, as shown in Fig. 5. This result indicates that EMD contains BMP-6-like molecules.



**Fig. 4** Effect of enamel matrix derivative (EMD) treatment on the expression of osteocalcin and type II collagen genes

C3H10T1/2 cells were cultured with 0, 10, 50, or 100 µg/ml EMD for up to 7 d. On day 1, 3, 5 and 7, the expression of osteocalcin and type II collagen mRNA was determined using real-time polymerase chain reaction, as indicated. The data shown are the mean±SD for three separate experiments. \* $P$ <0.05 EMD-treated versus control.



**Fig. 5** Western blotting of enamel matrix derivative (EMD) using anti-IGF-I and anti-BMP-6 antibodies

## Discussion

Although numerous studies have reported that EMD enhances osteoblastic and chondroblastic differentiation, the precise transcriptional mechanisms by which EMD produces these results were unknown. In the present study, we examined the effect of EMD by studying the expression of transcription factors that are involved in osteogenic and/or chondrogenic differentiation. Our central findings are that EMD induces expression of osteogenesis- and chondrogenesis-related transcription factors, and this effect is mediated by BMP-6-like molecules that are present in EMD.

Osteoblastic and chondroblastic differentiation is controlled by multiple transcription factors at various stages [12]. Generally, numerous studies or reviews demonstrated that Runx2, Osterix, Dlx5 and Msx2 were critical transcription factors for osteoblastic differentiation, and Sox5 and Sox9 were also crucial for chondroblastic differentiation [12–17]. Two novel zinc finger transcription factors, such as AJ18 [18] and Zfp60 [19] regulated osteoblastic and chondroblastic differentiation, respectively. Therefore, we focused on the above transcription factors in this study.

We examined the effect of EMD on the expression of osteogenesis- and chondrogenesis-related genes using real-time PCR. EMD treatment caused a significant increase in the expression of mRNAs encoding Runx2, Osterix, Dlx5, Msx2, and AJ18. Runx2, Osterix, and AJ18, all zinc-finger proteins, are transcription factors associated with osteoblast differentiation [18,20,21]. Runx2 has been identified as a direct inducer of osteocalcin expression [22,23], and deletion of Runx2 and Osterix causes a lack of ossification [21]. AJ18 was identified by a differential display of genes showing up-regulation by BMP-7 in cultured fetal rat calvarial cells, and it binds to the osteoblast-specific element 2 and modulates transactivation by Runx2 [18]. Two homeobox transcription factors, Dlx5 and Msx2,

appear to regulate the development of mineralized tissues, including bone cartilage and tooth [24–28]. Dlx5 expression is correlated with osteoblast differentiation, and maximal expression of Dlx5 occurs during the final stages of *in vitro* osteoblast differentiation, suggesting that Dlx5 could be involved in the maturation of the bone cell phenotype [29]. In contrast, Msx2 is predominantly expressed by proliferating osteoblasts and decreases upon terminal differentiation. In the present study, significant inductive effects of EMD on the mRNA levels of Runx2, Osterix, Dlx5, and Msx2 were observed in the C3H10T1/2 cell culture on day 1–7. These observations might indicate that EMD stimulates osteoblastic differentiation via the induction of mRNA of osteogenesis-related transcription factors. However, AJ18 showed a biphasic phenomenon during the culture period. Since the AJ18 mRNA level was highly increased during osteoblastic differentiation and decreased upon maturation of the osteoblast, EMD might mediate, in part, both osteoblastic differentiation and maturation.

EMD treatment also caused increased expression of mRNAs encoding Sox5, Sox9 and Zfp60, which are involved in chondrogenic differentiation. Numerous studies have demonstrated that Sox5 and Sox9 are important transcription factors that mediate chondrogenic differentiation [30–32]. Zfp60 mRNA was found to be transiently expressed, coincident with chondrocyte maturation and the expression of the Indian hedgehog and parathyroid hormone-related peptide receptor genes. Since overexpression of Zfp60 decreases chondrogenic differentiation, Zfp60 may regulate cellular hypertrophy [19]. AJ18 is also highly expressed in developing cartilage, where it may regulate not only osteoblastic but also chondroblastic differentiation by altering Runx2 activity [18]. In the present study, significant inductive effects of EMD on mRNA expression of Sox5 and Sox9 were observed in C3H10T1/2 cultured cells on day 1–7. Our results indicate that EMD promotes chondrogenic differentiation of C3H10T1/2 cells by increasing the levels of the transcription factors involved in chondrogenesis. Moreover, Zfp60 and AJ18 expression were also increased by EMD treatment. Thus, we conclude that EMD may regulate the amount of space that becomes filled by bone marrow through the modulation of Zfp60 and AJ18 expression.

In light of the above findings, we performed an immunoblotting analysis to show that Runx2 and Sox9 proteins are up-regulated by EMD. Our results indicate that EMD causes a substantial increase in the expression of Runx2 and Sox9 at the protein level.

To confirm EMD enhanced osteoblastic and chondro-

blastic maturation, we examined the expression of osteogenic and chondrogenic markers, such as osteocalcin and type II collagen, respectively. Osteocalcin, one of the markers of osteoblastic maturation [33], mRNA expression was significantly increased by the addition of EMD. Type II collagen, a typical chondrogenic marker [34], mRNA expression was also increased with EMD treatment. Our results suggested that EMD stimulates not only the transcription level but also the maturation stage.

Although we previously reported that EMD diverts the pluripotent mesenchymal cell line C2C12 to an osteogenic and/or chondrogenic differentiation pathway via BMP-2, BMP-4- or BMP-7-like molecules present in EMD [9,10], we suspected that EMD contains BMP-6-like molecules. Since BMP-6, but not BMP-2, was recently reported to strongly promote osteogenic/chondrogenic differentiation [35,36], we postulated that the ability of EMD to promote chondrogenic lineage was mediated by BMP-6-like molecules present in EMD. As expected, we found that the anti-BMP-6 antibody cross-reacted strongly with EMD at an apparent molecular weight of about 60 kDa, which is the expected migration for BMP-6 by SDS-PAGE. These results provide evidence that EMD contains BMP-6-like proteins of about 60 kDa. Any apparent size discrepancies are readily explained by the fact that the EMD used in the present study was prepared from fetal porcine tooth germs, not from bone cells, and could thus contain different isoforms [37–40].

In conclusion, EMD stimulates osteogenic and/or chondrogenic lineages of multipotent mesenchymal cells via induced expression of osteogenesis- and chondrogenesis-related transcription factors, and the ability of EMD to promote these effects may be mediated by BMP-like molecules, suggesting that EMD could be useful as a reagent for bone and cartilage regeneration.

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